# Cinemicrographic Study of the Development of Subsurface Colonies of *Staphylococcus aureus* in Soft Agar

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Cinemicrographic studies revealed that the development of elongated subsurface colonies of *Staphylococcus aureus* in soft agar (<0.2% agar) originated with a colony-forming unit of about 10 to 20 cells. It was then observed that small clusters of 3 to 12 cells broke off from the main colony unit and drifted away under the combined influence of gravity and Brownian motion. Once the downward or slightly sideward motion of the small clusters ceased, the clusters would continue to increase in size; at the same time, additional small clusters broke off, and the cycle was repeated until the entire colony was formed. Displacement and velocity measurements were made on the drifting small clusters. When compared with the dimensional growth rate and geometry of the subsurface colony, these showed that a correlation existed between the movement and velocity of the small clusters and the subsequent colony development. A relationship between the role of gravity reported in these results and the development of spherical colonies after rotation on a clinostat is suggested.

It was recently reported by Wilkins et al. (5) that the subsurface colonial morphology of *Staphylococcus aureus* grown in 0.18% agar, generally referred to as soft agar, could be altered by changes in the gravity field. For example, when growing cultures of *S. aureus* in soft agar were rotated about a horizontal axis by use of clinostat, which produced a uniform gravitational effect in the vertical plane, compact spherical colonies were demonstrated. The nonrotated control cultures contained typical elongated diffuse colonies which have been observed by a number of investigators (1–3). In an extension of this observation, a detailed study was made of the development of nonrotated subsurface colonies of *S. aureus* by use of phase-contrast, time-lapse cinemicrography. Selected areas of the filmstrips were then analyzed for colony growth rates, and the migration paths of cell clusters were charted and measured. This paper describes the manner in which subsurface colonies of *S. aureus* developed in soft agar with emphasis on the role that the displacement, velocity, and migration paths of clusters of cells played in this development.

## MATERIALS AND METHODS

**Bacterial strain and slide culture preparation.**

*S. aureus* ATCC 12600 was maintained on Trypticase soy agar (Bioquest) slants at 5 C. For the cinemicroscopic studies a culture slide chamber consisting of a stainless-steel ring, 1.65 cm in diameter and 0.25 mm deep, was affixed to a microscope slide with epoxy resin. Soft-agar medium prepared from 3% Trypticase soy broth (Bioquest) containing 0.16% agar-agar (Bioquest) was melted, cooled to 45 C, and inoculated with cells from a 24-hr culture grown in Trypticase soy broth (Bioquest). A thin coat of Vaseline was applied to the edge of the ring, and the well formed by the ring was filled to the top with the inoculated medium. Each well contained a final concentration of about four to six cells of *S. aureus*. A cover glass was then placed on the ring and held in position with Vaseline.

**Phase-contrast, time-lapse cinemicrography.** A research microscope (Zeiss, Oberkochen, West Germany; model WL) was mounted in the horizontal position to record the vertical and horizontal growth of the *S. aureus* colony by means of time-lapse photography. A warm stage (Zeiss) operating at 37 ± 1 C was used to maintain incubation temperature. All slide culture chamber preparations were examined with phase-contrast objectives in the range of 40 to 1,250 times magnification. Microscopic observations were recorded on Eastman Kodak Tri-X 16-mm black and white reversal film 7278 (ASA 160) by time-lapse with the use of a cinephotomicrographic apparatus (Sage Instruments, White Plains, N.Y.; series 503) and a 16-mm Bolex (Paillard Switzerland) cinema

camera. Frame rates were 8, 20, 30, 40, or 60 per min with 0.25-sec exposure time on each frame.

For the low-power, 2.4 to 6.0 times magnification, cinemicrographic studies, flat-walled tissue culture flasks (Bioquest) containing 19 ml of inoculated soft agar were mounted in the vertical position on the stage of a dissecting microscope (Wild, Heerburg, Switzerland; model M5). A prism was positioned between the objective and the tissue culture flask to permit time-lapse recording of the colony growth in the vertical position. Incubation temperature was maintained by an air curtain incubator (Sage Instruments, White Plains, N.Y.; model 279), operating at $37 \pm 1$ C. Single frames were exposed at rates of either 1 or 1.5 per min. In a few cases, black and white 16-mm film of ASA 40 was used in place of the ASA 160 film.

**Film data analysis.** After exposure, the film was developed in the usual manner for reversal-type film, and was then viewed on an x-y film-analyzing machine (Gerber Scientific Instrument Co., Hartford, Conn.; model 63-727) which enlarged the frames 35.5 times on the reading screen. The x-y position coordinates of the cluster centers were read from selected frames, formatted with time inputs, and put on data cards for computer analysis.

## RESULTS AND DISCUSSION

**Migrations of cell clusters.** Analysis of the cinemicrographic filmstrips revealed that a small cluster of cells was the basic building block of the *S. aureus* subsurface colony. The sequence of events during the early stages of colony development are shown in Fig. 1. Typically, after 6 hr of incubation at 37 C, the initial colony unit would grow to about 10 to 20 cells without interference, after which the cluster's growth would be randomly interrupted by the breaking off of small clusters containing 3 to 12 cells which drifted away under the combined influence of gravity and random Brownian motion. The role of these clusters in early colony development revealed that the colony was actually composed of a main cluster of cells near the point of origin, with other very small clusters randomly distributed below it as shown in Fig. 1. These small clusters were observed to break away from the periphery of the large cluster and drift downward or sideways, or both, a short distance through the culture media; in more advanced stages, a large number of cell clusters would fall away from the large upper cluster and fill in more of the area below the main cluster. Under increased magnification, the individual cells of these small clusters were distinguishable, and, although tenuously attached to one another at one or more points, they were observed to wiggle vigorously about these points of attachment

as the cluster was rolled and moved about by Browian motion. Once the downward or sideward motion of the small clusters ceased, cell division continued and the cluster continued to increase in size; at the same time, additional small clusters broke off and the cycle was repeated until the entire colony was formed. In rare instances, large clusters were observed to break into segments, and these acted as independent drifting clusters.

The above types of cluster action occurred throughout the developing colony but more actively in the lower portions so that a continual front of new growth was developed. The front advanced downward under the dominating force of gravity, while Brownian motion produced horizontal spreading, although to a considerably lesser extent. The dominance of gravity was evidenced by the observation that very few small clusters broke away from the upper periphery of the large clusters. Careful measurements showed that the colony area of the original cell cluster does experience a small amount of upward expansion, but this is considerably less than the downward growth of the colony, as shown in Fig. 2, which also depicts the filmed outline of a two-dimensional growth of a typical subsurface colony.

**Measured motion of S. aureus clusters.** Migratory movements of the cell clusters, which were described in the previous section as the colony building block, were measured to gain quantitative information on the role of cell clusters in colony development. In this section, velocities, settling times, distances, and directions of movements of the cell clusters are described.

The motions of *S. aureus* clusters in soft agar were analyzed from the time-lapse film data, and the motions considered were translational movements of the geometric centers of the *S. aureus* clusters. These analyses began at the time and point of physical separation of the cluster and ended with termination of cluster movement. The data rate at which these motions were read varied, but averaged about 5 to 10 points per min except for cluster no. 3 in Fig. 3 to 8, in which 60 measurements per min were made. The detailed data shown on cluster 3 illustrate that the clusters were undergoing more activity than shown by the graphs of clusters 1, 2, and 4, which, in effect, show more averaging of the real time motions. However, we considered the averaged cluster motion sufficiently detailed to assess the contributions of these clusters to overall colony development.
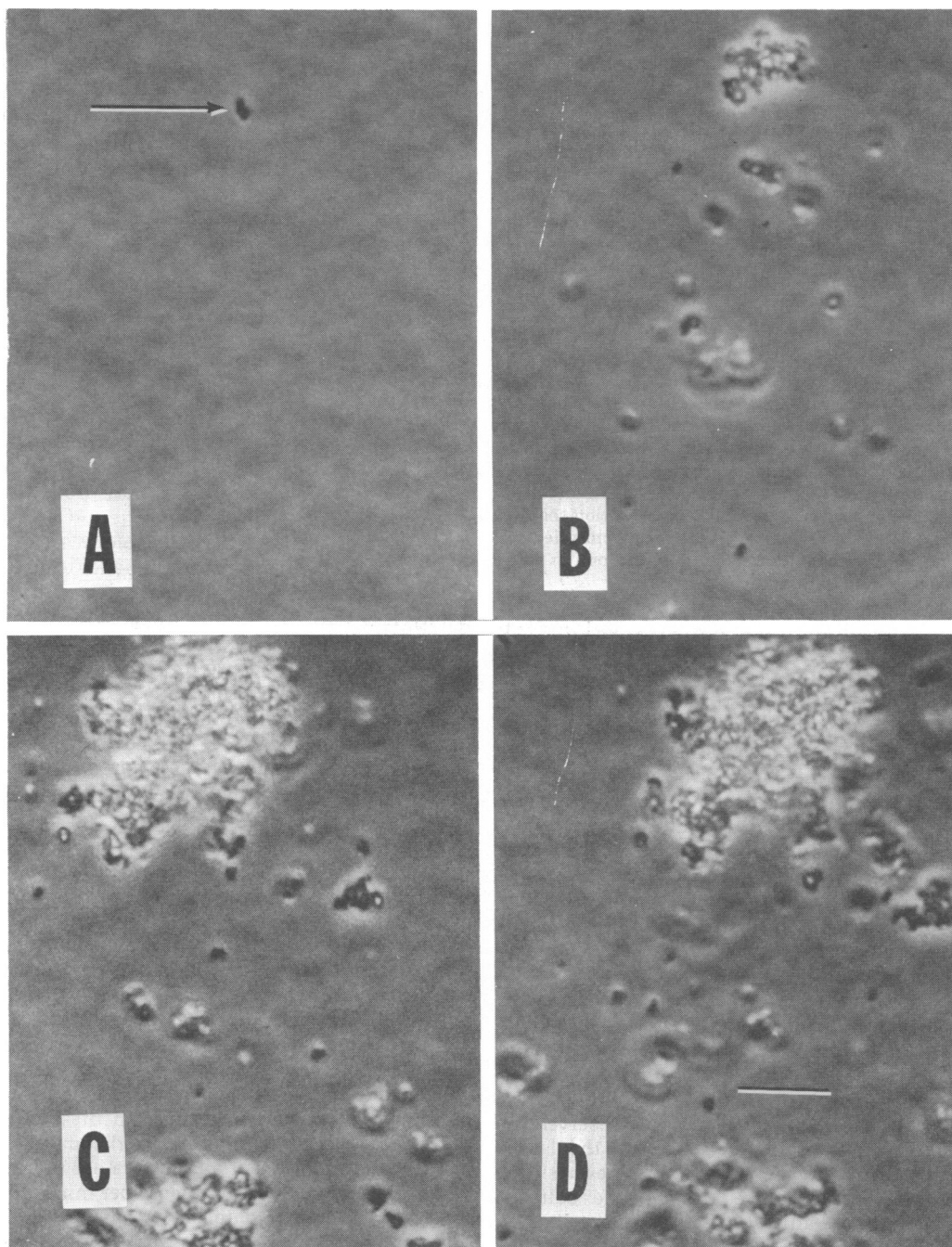
Trajectory data of four typical *S. aureus*

FIG. 1. *Development of S. aureus subsurface colony in soft agar. (A) Initial cluster of cells. (B) Separation of cell clusters from initial cluster. (C) Showering of cell clusters and development of secondary clusters. (D) Further development of primary and secondary clusters with continued release of clusters. Note that the downward colony growth is oriented toward bottom of figure. Marker bar equals 20 μm.*
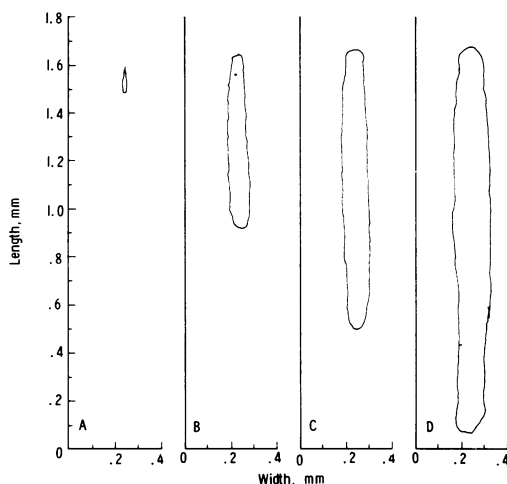
FIG. 2. *Sequential two-dimensional growth of a typical S. aureus subsurface colony in soft agar after (A) 6 hr, (B) 18 hr, (C) 30 hr, and (D) 40 hr of incubation at 35 C.*

clusters are presented in Fig. 3 in which the zig-zag path resulted primarily from Brownian movements exerted on the clusters. The displacement history of the four clusters, when resolved into vertical and horizontal components (Fig. 4 and 5), also demonstrated the erratic, unpredictable effects of Brownian motions coupled with gravitational settling. In Fig. 6 are shown the velocity of these motions in the vertical plane, with the upward direction as positive and downward as negative. Likewise, the horizontal velocities, with positive values indicating motions to the right and negative to the left, are shown in Fig. 7. All of the foregoing velocity values were derived from the point-to-point displacements with time along the respective trajectories; successive data points for these clusters were 6 sec or less apart. The horizontal and vertical components of velocity were combined in Fig. 8 and show the total velocity of each cluster along its trajectory without regard to its direction.

It has been shown in the trajectory plots for *S. aureus* that from its breakaway point the cluster followed a very indirect path to its final point of entrapment. The straight-line distance between these two points or the resultant total displacement accomplished by the cluster, however, is what determined its contribution to the dispersion and spreading of the cells and the eventual development of the overall colony. The total displacements of 38 *S. aureus* clusters are shown in Fig. 9, in which the origin for these analyses was defined as that point where the clusters broke away and began to move. These

straight-line displacements, when resolved into vertical and horizontal components, indicated the relative resultant direction of travel of the clusters. The ratio of vertical to horizontal displacement averaged 4.4 for the 38 clusters. Further, the vertical and horizontal components of a cluster's displacement, divided by the settling time, provided effective velocity components for the resultant displacement. The averaged data for 38 clusters are summarized in Table 1.

Using the ratio of average vertical to average horizontal movement of clusters, we considered it of interest to determine whether a correlation existed between this ratio and that for the length to width measurements of developing colonies. For comparison, the histories of the ratios of colony length to maximal diameter for five *S. aureus* colonies are shown in Fig. 10, along with a line at 4.4, viz., the ratio of average vertical to average horizontal motion of *S. aureus* clusters. These results show that, although the average ratio of length to maximal diameter for the five colonies was somewhat higher than the ratio of average vertical to average horizontal displacement for the 38 clusters, a correlation does exist between averaged cluster motions and the resulting colonial geometry.

The average values of displacement and velocity described for *S. aureus* clusters were then compared with the dimensional growth rate and geometry of the subsurface colony in soft agar (Fig. 2). The data supported the hypothesis that colony geometry and rate of development were governed by the movement and velocity of the cell clusters.

**Role of agar concentration and implications to clinostat observations.** The results of these cinemicrographic studies not only provided an explanation for the development of elongated subsurface colonies of *S. aureus* in soft agar under a gravity-oriented axis, but at the same time they cast some light on the clinostat studies (5) in which spherical colonies developed after exposure to a uniform gravity field. As pointed out, the elongated colony developed from small cell clusters which broke away from a larger cluster and, under the influence of gravity, migrated to a new area where the cluster increased in size. Small clusters again broke away and the cycle was repeated; in essence, an elongated colony was composed of a large number of microcolonies. The reasons why the cell cluster movement was terminated are not clear, but certainly must be related to the soft agar which, at the slightly gelled concentration of 0.18%, provided suffi-
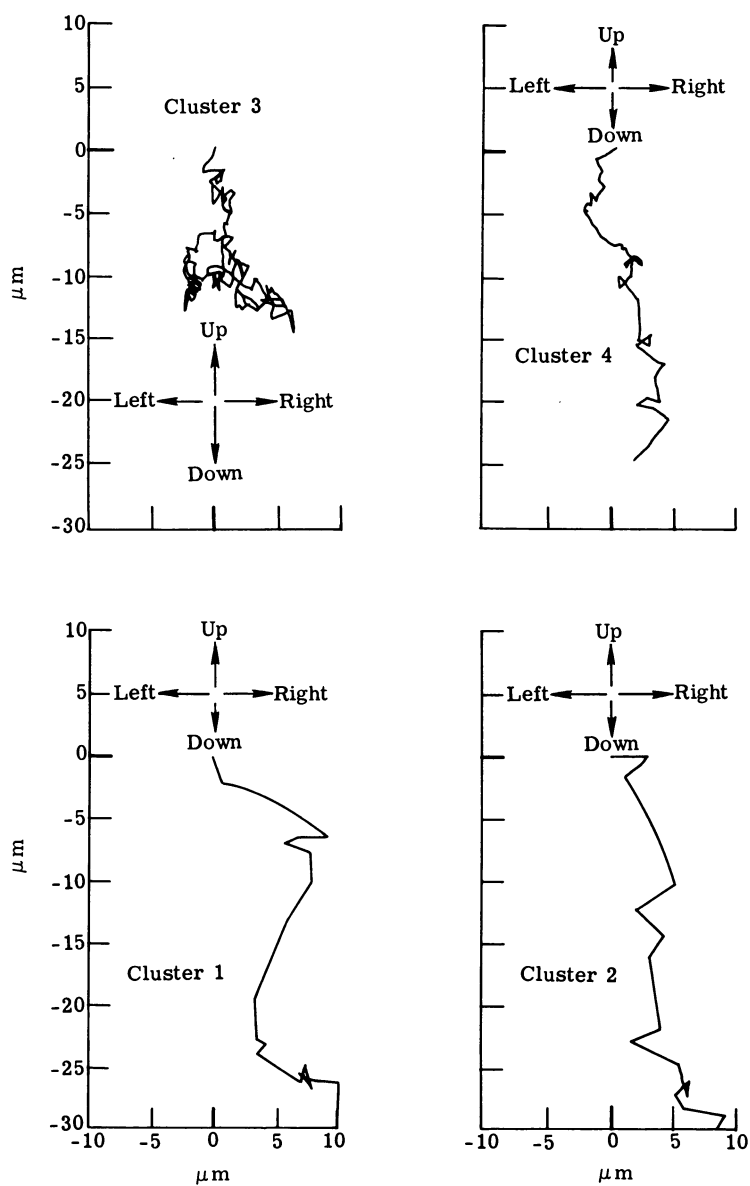
FIG. 3. *Migration paths of S. aureus clusters in soft agar. The origin represents the breakaway point of the cluster.*

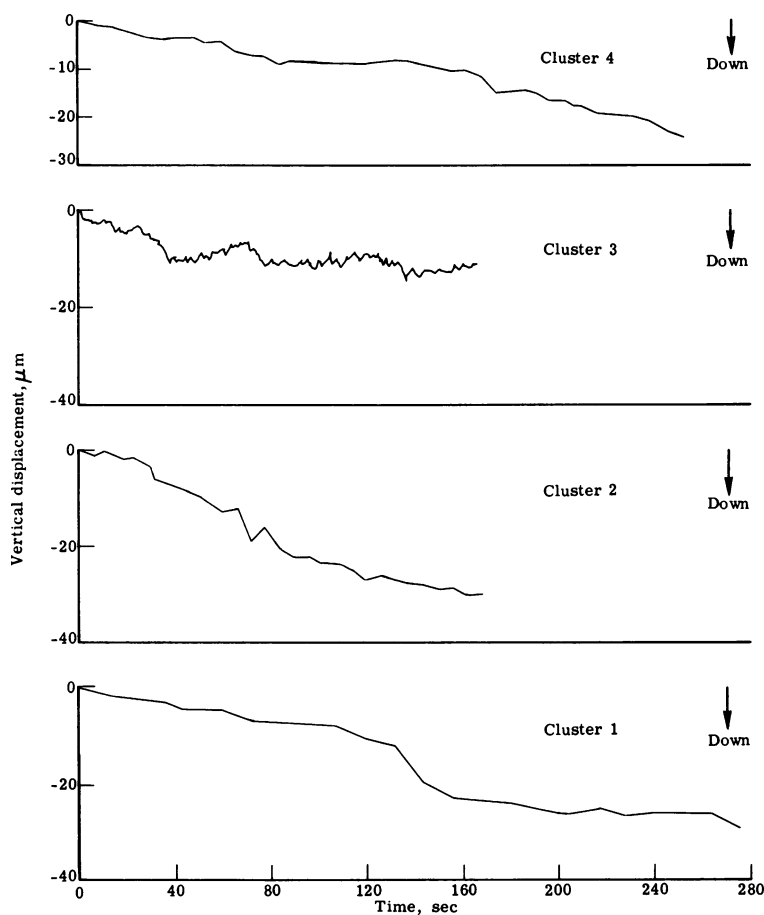FIG. 4. *Variation of vertical displacement of S. aureus clusters with time. Displacements downward were considered as negative and upward as positive.*
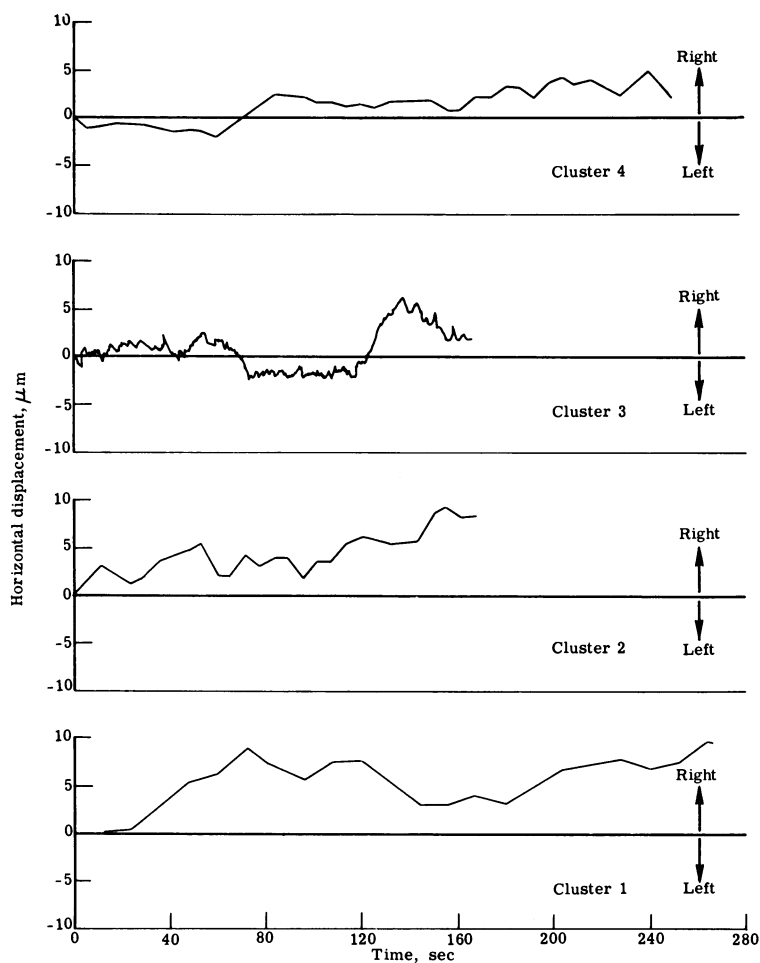
FIG. 5. *Variation of horizontal displacement of S. aureus clusters with time. Displacements to the left were considered as negative and to the right as positive.*
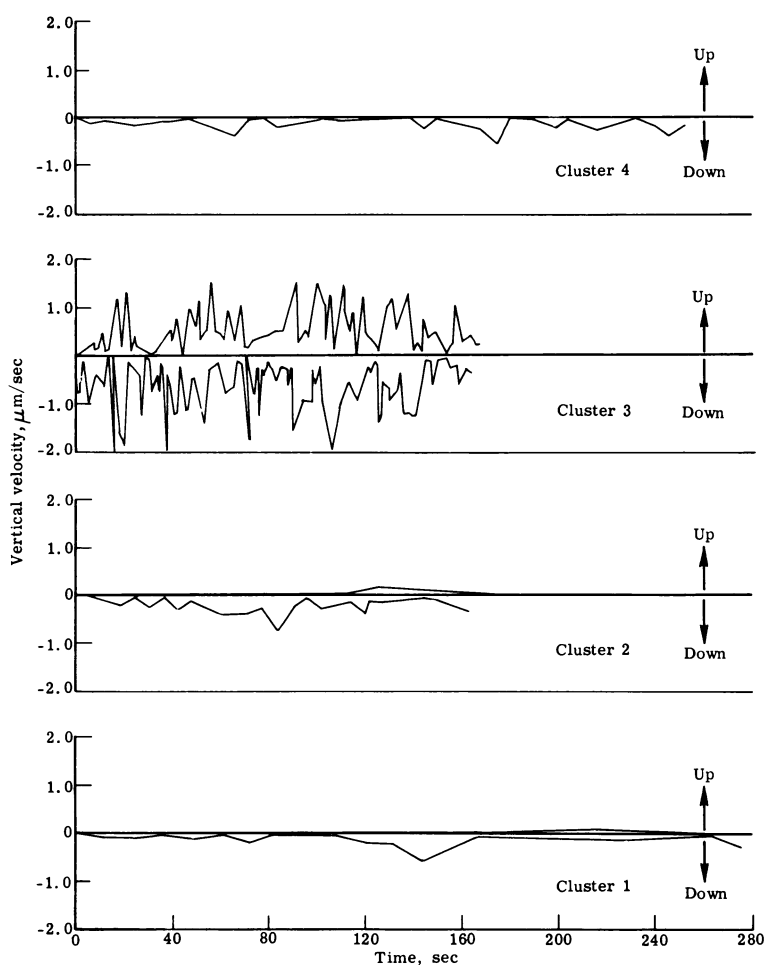
FIG. 6. *Variation of vertical velocity of S. aureus clusters with time. Upward motions were considered as positive and downward as negative.*
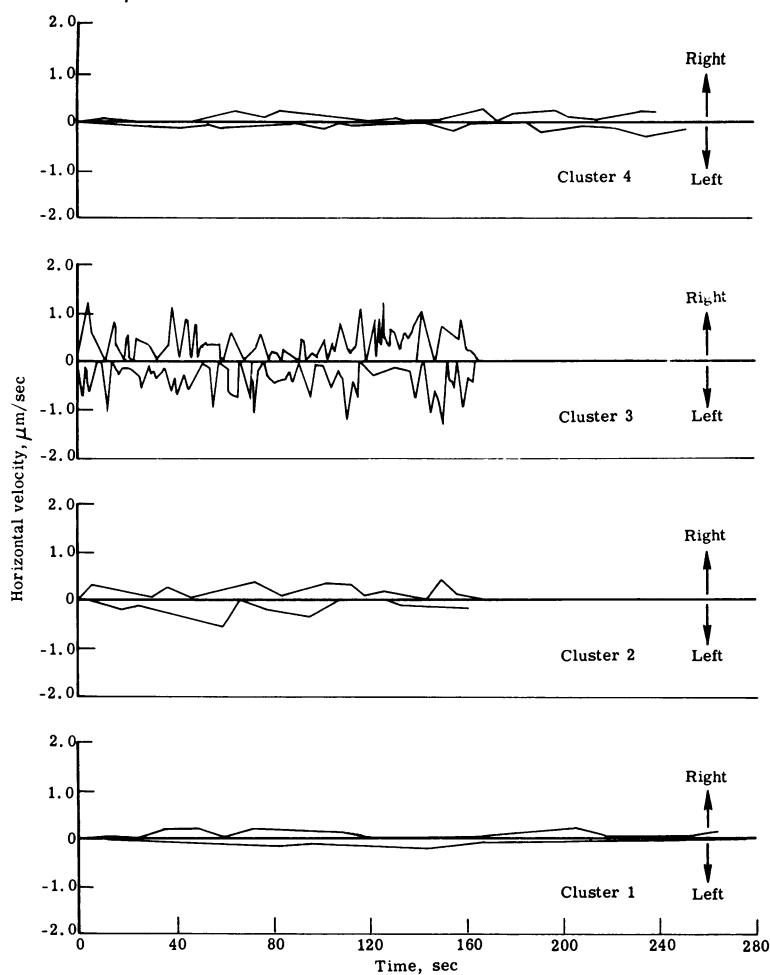
FIG. 7. *Variation of horizontal velocity with time. Motions to the left were considered as negative and to the right as positive.*
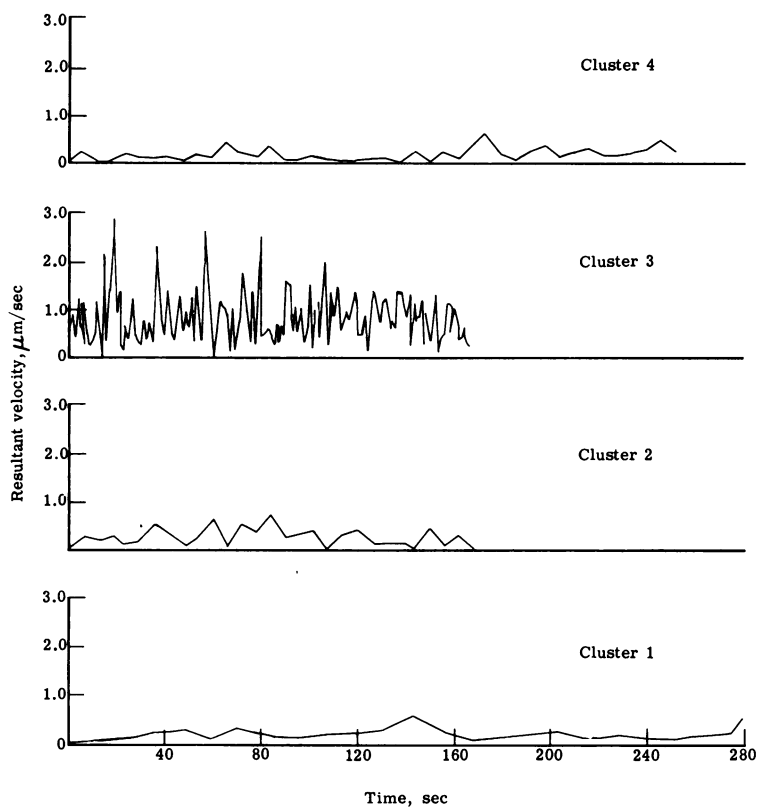
FIG. 8. *Variation of resultant velocity of S. aureus clusters with time. Resultant velocity was measured along the trajectory of the cluster.*
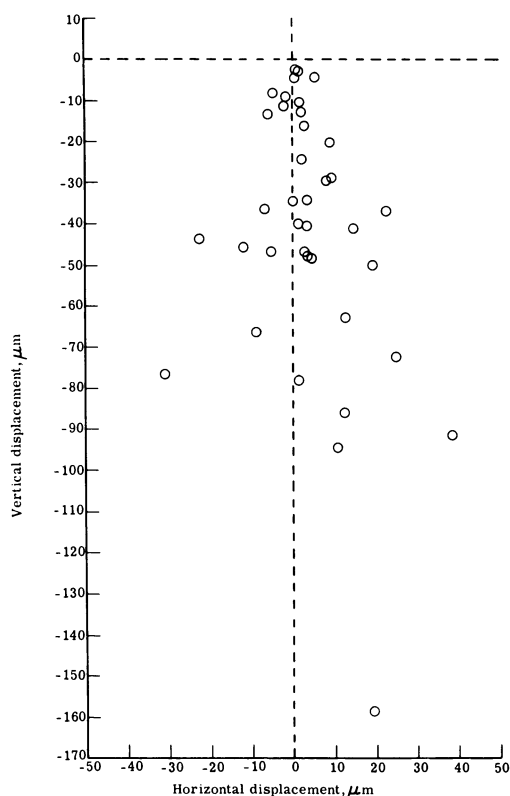
FIG. 9. *Resultant displacement of S. aureus clusters.*

cient suspension for the colony and at the same time permitted movement of the clusters. Although one can only speculate on possible mechanisms, it appears reasonable to consider soft agar as a linked network of fibrous molecules which are nonuniformly distributed throughout the medium, resulting in clots of gelled molecules in a less gelled fluid; it is these clots which could be the entrapment points for the migrating cell clusters. Although this point

TABLE 1. *Migration data results for Staphylococcus aureus clusters*

| Parameter | Avg values for 38 clusters |
|---|---|
| Settling time[a] .............. | 17.3 min (1,021 sec) |
| Resultant horizontal displacement ..................... | 9.0 μm |
| Resultant vertical displacement ..................... | 39.5 μm |
| Resultant total displacement . | 40.5 μm |
| Horizontal velocity required to reach end point by direct path .................... | 0.017 μm/sec |
| Vertical velocity required to reach end point by direct path .................... | 0.072 μm/sec |
| Total velocity required to reach end point by direct path ... | 0.072 μm/sec |

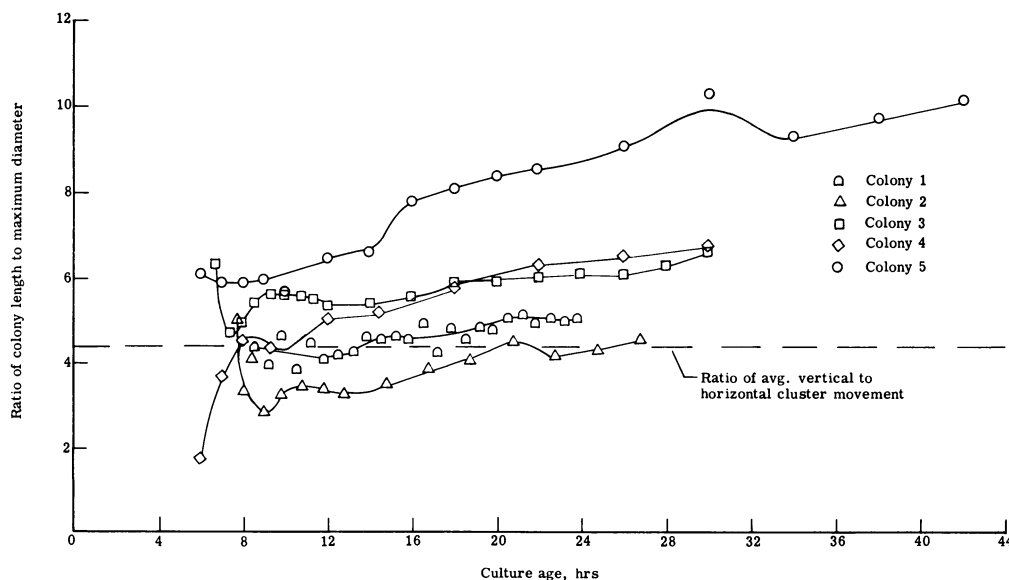[a] Time between cluster separation and termination of movements.



FIG. 10. *History of ratio of length to maximal diameter of S. aureus colonies in soft agar.*

has to be confirmed, it is in general agreement with some of the known physical characteristics of agar (4).

It is clear from these studies that the predominant force which governed the development of the elongated subsurface colony was the action of gravity on the cell clusters. It follows, therefore, that if the effect of gravity were made uniform in the vertical plane, as in the clinostat experiments (5), then the cell cluster movements in the vertical plane normally due to gravity would cease. Rather, it is reasonable to assume that under clinostat conditions the initial cluster of cells would grow to a given size, at which time one of two events could take place: (i) a cluster of cells could break off due to Brownian motion or some other effect and, in the absence of gravitational settling, would remain essentially in place and continue dividing, or (ii) the initial cluster would not develop separate clusters and continue growing as a unit. In either event, the end result would be a spherical-type growth rather than the elongated, diffuse colony observed under a gravity-oriented axis. Experiments are being planned for future space missions to examine the development of subsurface colonies of *S. aureus* and other bacteria in the absence of gravity.

## LITERATURE CITED

1. Alami, S. Y., and F. C. Kelly. 1959. Demonstration of staphylococcal clumping factor and free coagulase in soft agar media. J. Bacteriol. **78**:539–544.
2. Alami, S. Y., R. J. Merriman, and G. J. Race. 1968. Further evaluation and use of the soft agar medium for the simultaneous demonstration of mannitol fermentation, clumping factor, and free coagulase production by staphylococci. J. Clin. Pathol. **50**:517–524.
3. Finkelstein, R. A., and S. E. Sulkin. 1958. Characteristics of coagulase positive and coagulase negative staphylococci in serum-soft agar. J. Bacteriol. **75**:339–344.
4. Hickson, T. G. L., and A. Polson. 1968. Some physical characteristics of the agarose molecule. Biochim. Biophys. Acta **165**:43–58.
5. Wilkins, J. R., M. B. Hatten, and S. Salmirs. 1969. Effect of gravity on the colonial morphology of staphylococci in soft agar. Appl. Microbiol. **18**:680–681.